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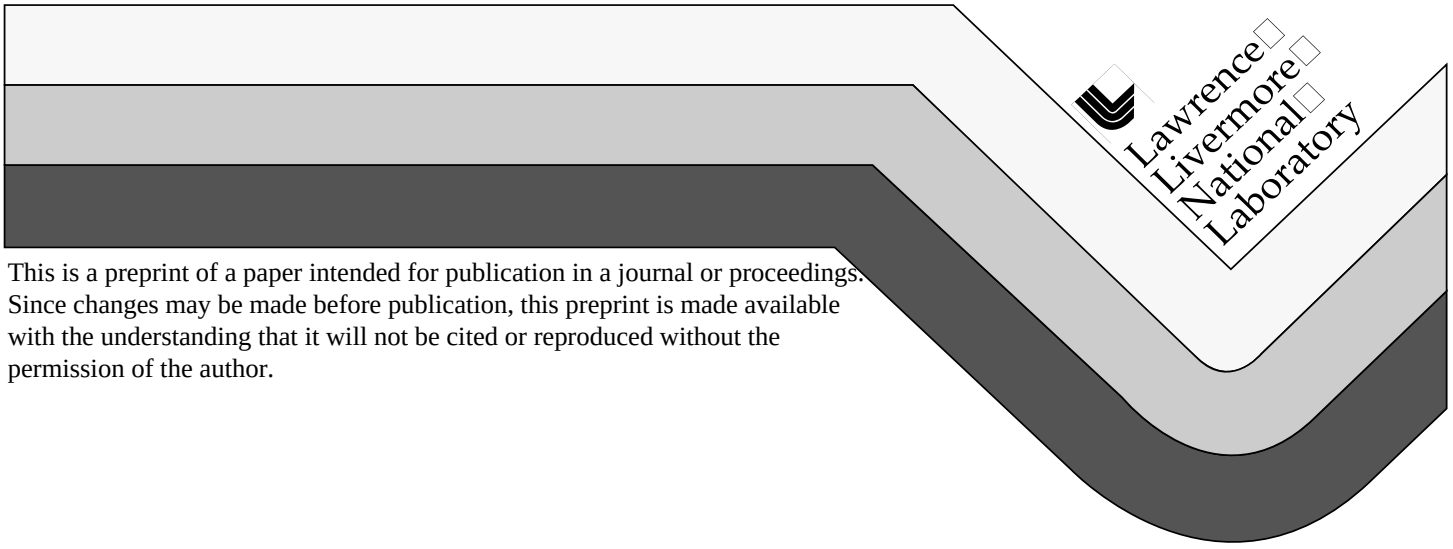

INVESTIGATION OF BACTERIAL TRANSPORT IN THE LARGE-BLOCK TEST, A THERMALLY PERTURBED BLOCK OF TOPOPAH SPRING TUFF

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ABSTRACT

This study investigates the transport of bacteria in a large, thermally perturbed block of Topopah Spring tuff. The study was part of the Large-Block Test (LBT), thermochemical and physical studies conducted on a 10 ft × 10 ft × 14 ft block of volcanic tuff excavated on 5 of 6 sides out of Fran Ridge, Nevada. Two bacterial species, *Bacillus subtilis* and *Arthrobacter oxydans*, were isolated from the Yucca Mountain tuff. Natural mutants that can grow under the simultaneous presence of the two antibiotics, streptomycin and rifampicin, were selected from these species by laboratory procedures. The double-drug–resistant mutants, which could be thus distinguished from the indigenous species, were injected into the five heater boreholes of the large block hours before heating was initiated. The temperature, as measured 5 cm above one of the heater boreholes, rose slowly and steadily over a matter of months to a maximum of 142°C. Samples (cotton cloths inserted the length of the hole, glass fiber swabs, and filter papers) were collected from the boreholes that were approximately 5 ft below the injection points. Double-drug–resistant bacteria were found in the collection boreholes nine months after injection. Surprisingly, they also appeared in the heater boreholes where the temperature had been sustainably high throughout the test. These bacteria appear to be the species that were injected. The number of double-drug–resistant bacteria that were identified in the collection boreholes increased with time. An apparent homogeneous distribution among the observation boreholes and heater boreholes suggests that a random motion could be the pattern that the bacteria migrated in the block. These observations indicated the possibility of rapid bacterial transport in a thermally perturbed geologic setting. The implications for colloid transport will need to be reviewed.

INTRODUCTION

Transport of bacteria in fractured rocks and sediments is an important factor that determines the capability of long-term operation of a high-level nuclear-waste repository. Bacterial migration in fractured rocks and sediments in a repository can accelerate the transport of radionuclides. For example, radionuclides can adsorb to the extracellular polysaccharide of bacterial cells; it can also chelate with cell membrane that bears electric charge [1, 2]. In addition, metabolism of bacteria can alter the pH in the environment, which could increase the solubility of radionuclides and facilitate their transport. Bacteria can also act as colloids. The effects of bacteria on a repository can also the positive. Bacteria can carry out redox reactions that could reduce the solubility of radionuclides and, thus, could immobilize them in the repository [3].

This study investigates the transport of bacteria in a large, thermally perturbed block of Topopah Spring tuff. This research is part of the Large-Block Test (LBT), thermochemical and physical studies conducted on a 10 ft × 10 ft × 14 ft block of volcanic tuff excavated on 5 of 6 sides out of Fran Ridge, Nevada [4].

EXPERIMENT

Two bacterial species, *Bacillus subtilis* and *Arthrobacter oxydans*, were isolated from the Yucca Mountain tuff. Natural mutants that can grow under the simultaneous presence of the two antibiotics, streptomycin and rifampicin, were selected from these species by laboratory procedures [5]. The characteristics of double-drug resistance distinguish the natural mutants from the

indigenous species. The mutants were cultured in nutrient broth that contained 0.5% agar at 30°C for 3 days and then were injected in that medium into the 5 heater boreholes of the large block hours before heating was initiated. The procedures for the culturing of these two double-drug–resistant bacteria and installation of them in the large block have been described [4, 5].

To collect bacterial samples from the observation boreholes, a piece of cotton cloth (which can adsorb bacterial cells) was inserted the length of each of the three boreholes (NO1, NO2, and EO3) that were approximately 5 ft below the injection (heater) ports. These installed cotton cloth strips stayed in the boreholes until the next sampling, at which time they were removed and replaced with new ones.

Alternatively, bacterial cells were collected with a swab of glass fiber (or a piece of filter paper). A Pyrex™ glass tube, which was also installed in the collection bore holes was pulled out of the ports with the pieces of cotton cloth. The tube was swiped with a moistened sterile glass-fiber swab (or a filter paper). The swabs (or filter papers) were then subjected to bacterial assays.

Cells that were possibly present in the heater boreholes were collected after the entire LBT experiment was completed. The heaters were removed from the block, and their surfaces were swiped with moistened sterile filter paper.

Cells on the bacterial samples (cotton cloth, glass-fiber swab, or filter paper) were enumerated by patting the samples on freshly prepared R2A agar plates that contained streptomycin and rifampicin. The concentrations of streptomycin and rifampicin in the agar plates were 30 mg/l and 170 mg/l, respectively. Each sample was cultured in duplicate plates. The plates were then incubated at 30°C for 3 days.

RESULTS

Hours after installation of the bacteria, the heaters of the large block were turned on, and the temperature of the large block rose slowly and steadily over a matter of months to a maximum temperature of 142°C (Fig. 1).

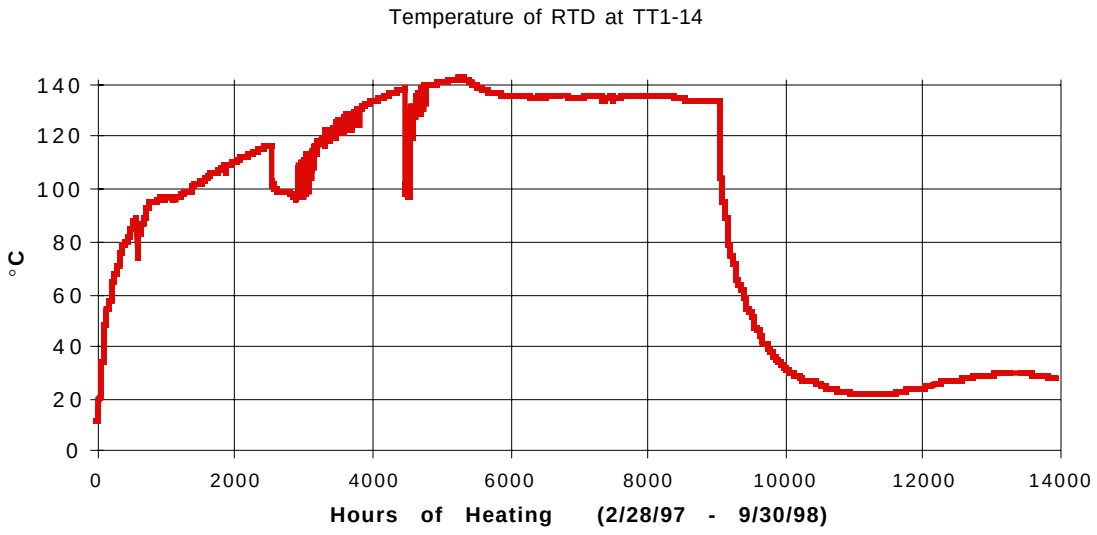

Figure 1. Temperature measured at one point 5 cm above one the heater holes, TT1-14, as a function of time. The temperature increased to approximately 142°C before the heaters were turned off at day 389 (9336 hours).

The bacterial samples were collected from the observation boreholes NO1, NO2, and EO3 at days 172, 271, 354, 404, 510, and 595 after injection of the cells. The heaters were turned off at

day 389, and the temperature cooled slowly. Double-drug–resistant bacteria were found in the collection (observation) boreholes 9 months (day 271) after their installation. Figure 2 shows the numbers of bacterial colonies developed on the double-drug plates, cultured with cotton cloth samples from the various collection boreholes, as a function of time. The number of double-drug–resistant bacteria that were identified in each borehole increased with time up to day 404, and no double-drug bacteria were observed at day 510 and day 595. There appears to be consistency among the three profiles of cell number versus time that represent the three boreholes (Fig. 2); all have the highest cell number observed at day 404. In addition, more cells were found at NO1 than at any other boreholes (Fig. 2).

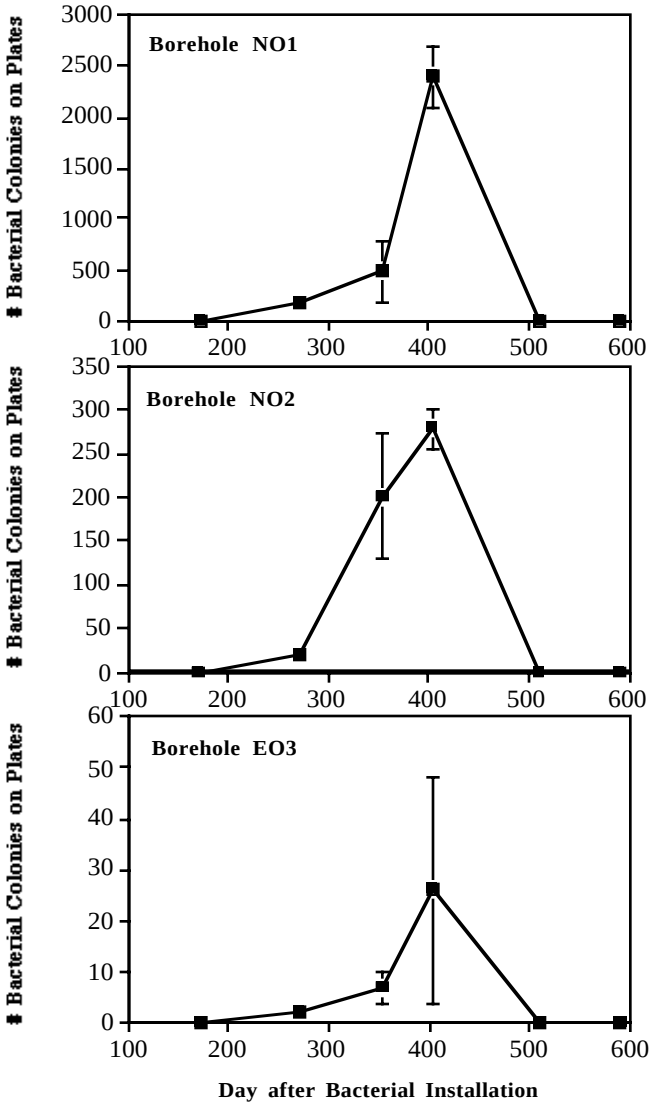

Figure 2: Number of double-drug–resistant bacteria present in the various collection boreholes (NO1, NO2, and EO3) as a function of time, collected with the cotton cloth strips. Means and standard errors shown in those plots were derived from bacterial counts of duplicate double-drug plates. Data points where error bars are not presented indicate that errors are smaller than or equal to the size of the symbol.

No double-drug–resistant cells were identified on the glass-fiber samples up to day 354. To explore if the sampling method affects the recovery of double-drug cells from the boreholes, after day 354 filter papers were used to replace glass-fiber swabs to swipe the Pyrex glass tube from the boreholes. Surprisingly, numerous cells were found with the new collection method. At day 404, the numbers of double-drug cells identified on the filter papers from NO1, NO2, and EO3 were 2600 ± 150, 221 ± 5, and 2400 ± 300, respectively. It should be pointed out that the number of double-drug cells identified on the filter paper at EO3 (2400 ± 300) was significantly higher than that identified on the cotton cloth for the same borehole (26 ± 21, Fig. 2).

In addition to examining the collection boreholes, the heater boreholes were also examined for double-drug bacterial cells after the LBT experiment was completed. Presence of double-drug cells in the boreholes could represent cells that were remaining there after their injection, or it could imply the migration of cells back to the boreholes after a long period of time of traveling. The results (Table I) indicate that double-drug cells were found in all five heater boreholes (EH1 through EH5).

Table I. Double-drug–resistant bacterial cells found at the various heater boreholes. Duplicate plates were done for each EH filter-paper sample. The double-drug R2A plates were incubated at 30°C for 3 days after being inoculated (patted) with the entire filter-paper sample in the laminar-flow hood.

Borehole	# Bacterial Colonies on Plate	Colony Description
EH1	13	1 big yellow (3 mm), others white .8-2 mm
	12	1 big yellow (2.5 mm), others white .8-2 mm
EH2	15	2 yellow (~2 mm), others white .8-2 mm
	13	all white .8-2 mm
EH3	16	all white, .8-2 mm
	9	1 yellow ~2 mm, others white .8-2 mm
EH4	1	yellow 3 mm
	1	yellow 2 mm
EH5	1	yellow 3 mm
	3	all white .8-1.5 mm

Some representative bacterial colonies on the double-drug plates (both cotton cloth and filter paper isolates) were selected, based on their morphology, for further identification. A total of 741 colonies were picked from the double-drug plates. Ten colonies, numbered D404-NO1-F001 through -F005 and D404-NO2-C001 through -C005 (D = day, F = filter, C = cotton cloth), were tested for their capability to grow in the presence of 2% (20 g/liter) NaCl. Because *B. subtilis* can grow on 2% NaCl (up to 7%), and not many other bacterial species can tolerate salt concentration that high, this provides a simple, first-step identification of these isolates. Nutrient broth (0.8%) was used as the base medium. The results are summarized in Table II. Eight of the 10 initially picked double-drug–resistant colonies grew on 2% NaCl. The original double-drug strain, *B. subtilis*, also grew on 2% NaCl as well as on the base medium without salt. The *A. oxydans* double-drug mutant, on the other hand, did not grow on 2% NaCl, but it grew very well on nutrient broth without the salt (Table II). These results suggest a possibility that the eight salt-tolerant double-drug isolates (Table I) could be the *B. subtilis* strain we injected. Further tests that employed an even higher NaCl concentration, up to 7%, are underway. Carbon-source utilization experiments and other biochemical tests also will be performed to complete the identification of these LBT double-drug isolates.

Table II. Growth of LBT double-drug isolates on 2% NaCl. The bacterial isolates were cultured in a 15-mL test tube containing 5 mL of the medium.

LBT Double-Drug Isolate*	Medium	Growth**
D404-NO1-F001	0.8% nutrient broth + 2% NaCl	+
D404-NO1-F002	"	+
D404-NO1-F003	"	+
D404-NO1-F004	"	++
D404-NO1-F005	"	+
D404-NO2-C001	"	+
D404-NO2-C002	"	+
D404-NO2-C003	"	–
D404-NO2-C004	"	+
D404-NO2-C005	"	–
B. subtilis (strep^r rif^r)	"	+
B. subtilis (strep^r rif^r)	0.8% nutrient broth only	+
A. oxydans (strep^r rif^r)	0.8% nutrient broth + 2% NaCl	–
A. oxydans (strep^r rif^r)	0.8% nutrient broth only	++

* D = day, F = filter paper, C = cotton cloth; each bacterial isolate (or original mutant strain) was cultured in duplicate test tubes.

NO1 and NO2 designate two collection holes at the base of the block.

** "–" = no growth. "+" = growth (increasing number of the "+" signs represents increasing abundance of growth)

DISCUSSION

The results of this bacterial migration study indicate a nearly homogeneous distribution of double-drug cells among the collection boreholes (Fig. 2). Cell sampling method appears to be affecting the recovery of double-drug cells from the boreholes. For borehole NO1, nearly the same number of cells was restored from the borehole with either cotton cloth or filter paper sampling method (2400 ± 300 cells for the cotton cloth sample and 2600 ± 150 cells for the filter paper sample). Regarding EO3, it appears that less double-drug cells (26 ± 21 cells at day 404) were obtained from the borehole than from any other boreholes when cotton cloth samples were employed (Fig. 2). But a higher number of cells (2400 ± 300 cells at day 404, see Results above) were found to be restored from EO3 when filter paper was used to collect cells. For NO2, less cells were recovered than from NO1 with either sampling method, but the number is only one order of magnitude lower than that restored from NO1.

At the height of the heating phase, a thermal gradient existed in the block from 110°C (and as high as 142°C, Fig. 1) at the heater, to near 60°C at the top and the base of the block. Thus, we expect that the installed bacteria may exhibit more than one response to the thermal pulse. It is well known that vegetative cells (cells that are metabolically active) cannot survive at temperatures greater than 110°C. If there were no blind spot nor any area in the block that were highly heat-resistant (low heat-transfer coefficient) where the cells could be protected, the cells in the block would have to transform into spores to better resist the adverse environmental conditions. Because *B. subtilis* can form spores, we expect to find them to survive. The salt-tolerant test results (Table I) support this. Eight of the 10 selected LBT isolates grew on 2% NaCl, a characteristic of our injected *B. subtilis*. However, the agar melts at 40°C and could have invaded fractures during the lengthy heating period, allowing *A. oxydans* to survive.

Double-drug cells were found not only at the collection boreholes but also appeared at the heater boreholes (Table II) where the temperature was significantly higher than at any other spots in the block. All these results suggest that the extremely high temperature conditions, such as those that will be encountered in a nuclear-waste repository, are not detrimental to some indigenous bacteria; thus, the indigenous bacteria might play an important role in the long-term operation of the repository. Despite the fact that bacteria could transform into spores and become dormant when the temperature of the repository rises, if the environment changed and temperatures cooled down, they could germinate and start their metabolic activity quickly, which might impact the stability of stored radionuclides.

If the bacteria exist as spores, their movement along the fractures of the block must be random (such as Brownian motion). The movement is then primarily determined by the aperture of the fractures and by the water or air flow. This suggests that the spores can exist throughout the block. The vegetative cells, on the other hand, move passively or by chemotaxis or by swimming with flagella. If the system were completely saturated with water, the movement from the heater boreholes to the collection boreholes (a distance of 5 ft) could take a matter of hours (or days at most). However, the system is not saturated. The first appearance of double-drug cells occurred nine months after their installation into the block (Fig. 2). After that, the numbers of cells found at the various collection boreholes increased with time (Fig. 2). Interestingly, similar densities of cells were recovered from the various collection holes (with cotton cloth strips and filter papers).

No double-drug–resistant cells were found at day 510 or day 595 (Fig. 2) after the heaters of the block had already been turned off for at least 4 months and the temperature in the entire block had started to cool. The results are surprising and intriguing; they might be related to the loss of moisture or heat gradient, which can create air currents or localized conditions in which the bacteria thrive. As the block is a thermally perturbed rock, the temperature of it increased with time, and the humidity in the fractures decreased [4]. Bacterial cells cannot migrate and continuingly accumulate at the collection boreholes if water is no longer available in the fractures. The weather could also be an important factor. From day 271 through day 404 (November 1997 through March 1998, the winter and spring seasons), when numerous cells were found at the collection boreholes, more rainfall was observed than was seen during the preceding months at the Yucca Mountain site where the LBT experiment was conducted. During the dry summer months following March 1998, the block had been covered, so the weather could not contribute to the absence of microbes at the end of the LBT studies. The results also suggest that no native double-drug–(streptomycin and rifampicin) resistant bacteria existed in the block.

SUMMARY

The migration of bacteria in a thermally perturbed geologic system has been demonstrated. Intriguing aspects of these results are the direct relationship between the thermal input and the observation of bacteria in the collection holes, and the presence of bacteria on the heaters themselves after the heaters were cooled and removed. The implication for colloid transport will need to be reviewed.

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